

THE FREQUENCY OF *MYTILUS EDULIS GALLOPROVINCIALIS* ALLELES IN WASHINGTON STATE MARINE WATERS WHERE THE SPECIES IS COMMERCIALY CULTIVATED

KENNETH M. BROOKS

Aquatic Environmental Sciences, 644 Old Eaglemount Road, Port Townsend, WA 98368

Comment [RE1]: Yellow highlighted sections in report indicate apparent missing or misspelled words or other edits needed, that the author should address.

ABSTRACT *Mytilus edulis galloprovincialis* has been described as an *invading nonindigenous species* introduced into the Pacific Ocean at some point in the past. A review of the published literature suggests that the taxonomic status of this mussel in the Pacific remains controversial and that substantive evidence of the introduction of *M. galloprovincialis* from the Mediterranean Sea to Southern California is lacking. Several studies have described the frequency of *M. e. galloprovincialis* alleles in feral populations of mussels from Puget Sound Washington. However, none of these studies were designed to randomly sample intertidal or subtidal populations of mussels and therefore, while there is ample evidence of the presence of *M. galloprovincialis* in numerous locations within Washington State, it is inappropriate to infer overall population characteristics based on previously published reports. Random samples collected in 1996, 1997 and 2002 in areas of intensive mussel culture *M. galloprovincialis* is found with a prevalence of one to two percent. Hybrids with *M. trossulus* generally represent zero to 4 percent of the population. However, ten percent of one sample of 30 mussels was hybrid.

Comment [RE2]: Missing word in this sentence?

INTRODUCTION

Origin of *Mytilus edulis galloprovincialis* like mussels in the Northeast Pacific. Numerous authors (Geller 1995; Hilbish 1999; Anderson 2002; Wonham 2004) have cited McDonald & Koehn (1988) a demonstrating that *M. galloprovincialis* is an introduced species in California. These authors make three references to this issue. In their abstract they state “Mussels in southern California are very similar to *M. galloprovincialis* Lamarck, 1819 from the Mediterranean Sea; it is probable that *M. galloprovincialis* was introduced accidentally to southern California.” In the discussion section they state, “The mussels from southern California are similar in allele frequencies to *Mytilus galloprovincialis* from the Mediterranean, a species that apparently has been introduced to several geographically widespread areas with relatively warm waters.” They go on to discuss the morphological similarities between San Diego and Mediterranean Sea blue mussels and state that, “Many marine species have been introduced to California (Carlton 1975), and it now appears that *M. galloprovincialis* can be added to that list.” Finally, in the systematics and distribution section, McDonald and Koehn (1988) state that, “The populations of *Mytilus galloprovincialis* in southern California, Japan, Hong Kong, and South Africa were apparently introduced.” It needs to be emphasized that the basis for McDonald and Koehn’s (1988) hypothesis was that the San Diego mussels were electrophoretically (at the 8 enzymes examined) and morphologically similar to mussels from the Mediterranean.

In contrast, Kafacov (1987 cited in Stewart *et al.* 1995) hypothesized that the common ancestor to the *M. edulis* species complex arose in the North Pacific during the early Eocene (40 to 50 million years before present) and subsequently dispersed into the Atlantic via the Bearing Sea. Sanjuan *et al.* (1997) substantiated that hypothesis and suggested that *M. galloprovincialis* evolved first in the Pacific Ocean and was distributed widely by a natural ancient trans-equatorial

Comment [RE3]: How did the referenced authors substantiate this hypothesis?

migration through the Pacific Ocean (Kafanov 1987; Koehn 1991; McDonald *et al.* 1991; and Vermeij 1991, 1992).

Kennington *et al.* (1995) presents an analytical assessment of this issue based on the analysis of small-subunit rRNA gene sequences observed in the *Mytilus* species complex and in *Geukensia demissa*. They identified six genetically distinct entities in the *Mytilus* species complex. Of particular interest in this discussion was their observation of greater genetic divergence (0.5% different alleles) between *M. galloprovincialis* from Washington State and the same species from France than between *M. trossulus* from Washington State and *M. galloprovincialis* from France. Divergence of the two *M. galloprovincialis* populations was 2.5 times greater than the divergence of *M. trossulus* from British Columbia when compared with *M. galloprovincialis* from France (0.2% different alleles). The authors concluded that because *M. galloprovincialis* from Washington State shared at most 99.5% of the 18S rDNA sequence with any other mytilid, it appeared to be misnamed and should be re-examined by comparing it with *M. edulis diegensis* as described by Coe (1946).

Life history of Mytilus sp. in the Northeast Pacific.

The Baltic mussel (*M. trossulus*) is found circumpolarly in cold water in the northern hemisphere and it is the dominant mussel in the Baltic Sea where salinities are reduced to 4 to 5 parts per thousand. Sarver and Foltz (1993) and Johannesson *et al.* (1990) have described *M. trossulus* as a euryhaline, cool-water mussel and *M. galloprovincialis* as a warm-water oceanic mussel (Hilbish *et al.* 1994). In the Atlantic, as one proceeds from low salinity to higher salinities and from northern latitudes to southern latitudes, the dominant siblings gradually shift from *M. trossulus* to *M. edulis* to *M. galloprovincialis* (Gardner 1996).

A similar pattern is observed in the Northeast Pacific with *M. galloprovincialis* dominating blue mussel populations below 41 °N latitude and *M. trossulus* dominating at higher latitudes. Within Washington State, Brooks (1991) found large numbers of *M. trossulus* in Saratoga Passage which experiences low winter and spring-time salinities due to freshwater inputs from the Fraser, Skagit and Samamish Rivers. Thirty-one of the 68 Washington State sites where healthy populations of *M. trossulus* were sampled to assess the prevalence of hemic neoplasia were sites that were heavily influenced by freshwater. Outside Saratoga Passage, *M. trossulus* was found intertidally only in the outwash of streams and rivers which provided a salinity refuge from starfish and other stenohaline predators which effectively control intertidal populations of blue mussels within most of the Pacific Northwest.

Brooks (1991), Heritage (1983) and Bower (1989) have reported increased mortality in *M. trossulus* at water temperatures above 10 °C with total loss of experimental populations at temperatures above 20 °C. Sea surface isotherms recorded by the US Department of Interior (USDI 1968) suggest that the current southern boundary of the distribution of *M. trossulus* is coincident with maximum oceanic temperatures of 14 to 15 °C. The intolerance of *M. trossulus* to higher temperatures makes successful colonization in southern California, where summertime oceanic temperatures exceed 18°C and temperatures in San Diego Bay are higher (US Dept. Interior 1968), unlikely. Based on these physiological constraints, it appears more likely that if *M. galloprovincialis* was introduced into southern California at some point in the past, there were no *M. trossulus* there to displace.

Brooks (1991) observed a significant population of *M. galloprovincialis* and hybrids with *M. trossulus* growing on piling in Dyes Inlet, which is a shallow, poorly flushed body of water

adjacent to the Bremerton Naval Shipyard. Summertime water temperatures in this bay approach 20 °C and the salinity was measured at ca. 28 ppt in both winter and summer. The *M. trossulus* were numerous and uniformly small, suggesting early senescence, whereas the *M. galloprovincialis* hybrids were much larger (~8 to 10 cm valve length) but fewer in number.

Matson *et al.* (2003) documented a partial gene-flow barrier between these two siblings associated with the winter (December through March) peak spawning of *M. galloprovincialis* and the spring (March and April) peak spawning of *M. trossulus*. However, both species produce small numbers of viable gametes at other times of year and there remains some potential for hybridization. Therefore, there are both environmental (low salinity versus high salinity) and reproductive partial barriers to hybridization. Winter temperatures in Puget Sound typically vary between 7 and 10 °C, which would likely result in long larval development times for *M. galloprovincialis* resulting poor recruitment of that species.

Previously reported frequencies of M. galloprovincialis alleles in Washington State mussel populations.

Mytilus galloprovincialis was first confirmed by Brooks (1991) in Washington State using protein electrophoresis. A total of nine enzymes were examined in 846 mussels from Washington State and 575 mussels from California, Oregon, Maine and Prince Edward Island. A diagnostic locus (*Pgm-2*) meeting the criteria of Avise (1974) was used to distinguish *M. trossulus*, *M. galloprovincialis*, and their hybrids. Feral mussels carrying *M. galloprovincialis* alleles were observed in Sequim Bay, Oyster Bay (Totten Inlet) and Dyes Inlet. All three of these waterbodies are relatively shallow; subject to high summer temperatures of ca. 20 °C; and are near potential sources of *M. galloprovincialis* larvae or adults. Sequim Bay is adjacent to major shipping channels in the Straits of Juan de Fuca and the John Wayne Marina. The Dyes Inlet population was found on piling at the west end of the bay, which is adjacent to the Bremerton Naval Shipyard and Oyster Bay lies at the head of Totten Inlet where *M. galloprovincialis* has been cultured since 1985. Sixty-seven percent of the mussels from Dyes Inlet that morphologically resembled *M. galloprovincialis* were found to be hybrids with *M. trossulus*. Approximately half of the remainder were *M. galloprovincialis* and half (18%) were morphologically misidentified *M. trossulus*. It should be emphasized that these were not random surveys. All of the nine mussels identified as *M. galloprovincialis* by shell morphology in Sequim Bay were hybrids.

Anderson *et al.* (2002) surveyed mussels at 33 sites in Puget Sound and Hood Canal by analyzing polymerase chain reaction (PCR) amplified Glu-5' gene and the internal transcribed spacer (ITS) locus described by Rawson *et al.* (1999). The surveys were not designed to randomly sample mussels, but the authors noted that they attempted to include both large and small mussels in their samples which varied between N = 16 and N = 64. *Mytilus galloprovincialis* genes were identified at 12 of the 32 sites from which feral mussels were collected with frequencies of 0.02 to 0.21. All of the mussels collected from the Totten Inlet raft cultures were *M. galloprovincialis*. Consistent with the conclusions of Rawson *et al.* (1999) who worked in California, Anderson *et al.* (2002) concluded that even where significant numbers of the two species are located sympatrically; hybrids were relatively uncommon and there was limited potential for introduced *M. galloprovincialis* to genetically pollute pre-existing populations of *M. trossulus*.

Wonham (2004) examined 390 *Mytilus* between 1997 and 2000 from public docks, marinas and a mussel farm using DNA analysis. The collections were reportedly made in a “haphazard” manner by selecting large (3 to 10 cm valve length) mussels that were visible and accessible among other fouling organisms on floating structures. Sixty-three percent of the Puget Sound mussels were homozygous for *Mytilus trossulus* alleles and 18% were *Mytilus galloprovincialis* homozygotes. A large proportion (24%) were hybrids.

Brooks (1996, 1997) conducted random and non-random surveys in Holmes Harbor, Washington which is tributary to Saratoga Passage. Holmes Harbor has been the site of a small scale raft mussel culture operation since the late 1980s. The 1996 survey was conducted prior to expansion of the facility and the 1997 survey represented the first year following expanded culture of *M. galloprovincialis*. The farm was subsequently closed due to high *M. galloprovincialis* mortality thought to result from low winter salinity conditions in Saratoga Passage. Non-random surveys included mussels collected in a shoreline search for individuals having morphological characteristics associated with *M. galloprovincialis*. In 1996, a total of 80 mussels resembling *M. galloprovincialis* were collected in Holmes Harbor. Seventy-seven of these were from June Beach. Fifty-three of the mussels in this non-random sample were analyzed electrophoretically revealing 26 *M. galloprovincialis*, 26 hybrids and one *M. trossulus*.

METHODS AND MATERIALS

Experimental design.

The results for non-random samples reported in Brooks (1996, 1997) demonstrate the ability to distinguish older (larger) *M. galloprovincialis* and hybrids from *M. trossulus* based solely on shell morphology. These apparent morphological differences demand that a random sampling design be employed if results are to be used to estimate population parameters. Non-random or haphazard sampling designs are useful for determining the presence-absence of *M. galloprovincialis* in Puget Sound and elsewhere, but they cannot be used to estimate the proportion of *M. galloprovincialis* genes in mixed populations of mussels.

In Holmes Harbor, a 3 meter square grid was established over intertidal populations of mussels at each sampling location. Samples were collected by randomly assigning Cartesian coordinates (x and y) within the grid and collecting all mussels lying within a 30 cm square quadrat centered on each randomly chosen point. The three samples were pooled and randomly subsampled to obtain 48 individuals for protein electrophoresis. Blind samples of known genotype were inserted into each cohort as a quality assurance check.

Random collections in Totten Inlet were made at the locations described in Figure 2. Very few mussels were found growing intertidally in sediment at any location within the inlet. Mussels were collected from predator fences; concrete oyster culture dikes and creosote treated piling. Systematic random samples were collected from these linear structures using a randomly selected starting point (from one end of the dike or predator fence or from the water’s surface on piling) followed by sampling at fixed intervals. All mussels were from a length of substrate sufficient to provide >60 mussels. Non-random samples involved the collection of ca. 30 mussels from each site with valves resembling *M. galloprovincialis*. These collections were made from structures (logs, piling, floats, concrete dikes, etc.) located outside the random sampling area.

1996-1997 protein electrophoresis.

Comment [RE4]: There needs to be a statement justifying and explaining the inclusion of the Holmes Harbor study in this report and clarifying that it was done prior to the Totten EIS study, but that the data from Holmes Harbor, and or the methods used in the Holmes Harbor study, are relevant to the Totten mussel raft EIS.

Comment [RE5]: The section under this heading describes methods and results from the author's cited PhD thesis (Brooks 1991) rather than methods or results from any 1996-1997 sampling, which were studies done by the author on another project. It appears that the author simply needs to move section titles and provide a consistent degree of section heading.

Brooks (1991) described three phosphoglucomutase (*Pgm*, E.C. 5.4.2.2) loci in all three sibling species. *Pgm-3* was rarely and faintly observed in healthy mussels. However, it became a dominant feature in phosphoglucomutase stained gels of mussels infected with hemic neoplasia. The other two loci (*Pgm-1* and *Pgm-2*) were reproducible in all populations. *Pgm-1*, with nine loci, was the first locus appearing after staining and appeared to be the locus reported by most researchers. *Pgm-2* was observable only after 45 minutes to one hour of incubation of stained gels at 37 °C. It met the requirements of Avise (1974) for a diagnostic locus (e.g. that the locus permitted correct identification of individuals, as to species or form, within a mixed population with a probability greater than or equal to 0.99). A total of 7 alleles were observed at *Pgm-2*. Three of these formed a subset of slow alleles associated with *M. trossulus* and the other four alleles formed a subset of fast alleles associated with *M. galloprovincialis* and *M. edulis*. Atlantic mussels from Booth Bay Harbor, Maine and Prince Edward Island, Nova Scotia were homozygous at *Pgm-2* for allele 6.

Only fresh tissues were used and the mussels were maintained on ice during the entire process. A small piece of digestive gland and another from the posterior adductor muscle were minced with forceps in 0.5 ml tris-HCL (pH 8.0) grinding buffer in pre-numbered, 1.5 ml microfuge tubes that had been frozen into a block of ice. After mincing, the tissues were sonicated at 75 watts for 10 seconds and spun down at 2,000 g for 10 minutes. The supernatant was absorbed onto two mm wide wicks for horizontal electrophoresis in 12.5% starch gels. The 28 cm long x 15 cm wide x 6 cm thick gels were run at 7.9 V/cm (21 watts) for 7 hours in tris-maleic buffer in a 4 °C cooler with a shaved ice pack applied to the top surface of the gel. Stains and staining methods are described by McDonald (1985). The stained gels were smoothly covered with Seran™ wrap to prevent desiccation during the one hour staining time required to observe *Pgm-2* at an incubation temperature of 37 °C. The gel mold was then placed back over the top of the stained gel; a glass plate placed on top; and the alleles transcribed onto a clear sheet of acetate. Known standards of Maine *M. edulis*, San Diego *M. galloprovincialis* and Hood Canal *M. trossulus* were frequently run with the unknown samples. The allele patterns found in comparing San Diego *M. galloprovincialis*, Hood Canal *M. trossulus* and previously identified hybrids from Silverdale, Washington are provided in Figure 1.

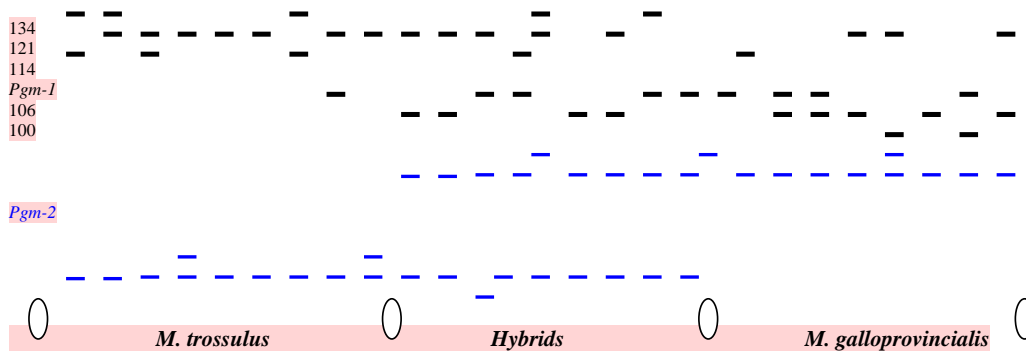


Figure 1. Reproduction of a phosphoglucomutase (*Pgm*) stained gel showing the relative migration of the *Pgm-2* locus which is diagnostic for Pacific coast populations of *M. trossulus* and *M. galloprovincialis* and a group of previously identified hybrids from Silverdale, Washington (Brooks 1991).

Comment [RE6]: This material would read better in the Discussion or Introduction section, rather than Methods section as it is not really a method used in this study, but information derived from prior studies that lays the basis for interpretation and discussion or the present studies.

2002 DNA analyses. In 2002, samples were held at 4 °C and shipped to the University of Delaware on ice via an overnight delivery service. The mussels were shucked; their adductor muscles excised and preserved in 70% ethanol for DNA extraction using a commercially available Qiagen DNAEasy kit. The *Mytilus* Glu gene coding for the *Mytilus* polyphenolic adhesive protein was amplified using the primer set JH5 / JH73 to yield a single 240 pb band in *M. trossulus* and a 202 bp band in *M. galloprovincialis*. These products were distinguished on an agarose gel (3% 3:1 NuSieve), providing identification of individual species as well as interspecific hybrids. The primers of Heath *et al.* (1995) were used to amplify a ~1250 pb DNA fragment spanning the *Mytilus* Intergenic Transcribed Spacer Region 1 (ITS-1), 5.8S rRNA and ITS-2 using the enzyme *Sau96 I*. The third locus examined targets an anonymous site derived from a *Mytilus edulis* genomic library, amplified by primers JH-2 and PR-9 (Rawson *et al.* 1996). The resulting amplicons are ~960 bp in all three blue mussel species and appeared to contain coding sequence adjacent to each primer, flanking a central intron. As described by Rawson *et al.* (1996), digestion of the amplicon with *Spe I* produces species-specific restriction patterns, with two patterns in *M. trossulus* and one in *M. galloprovincialis*.

RESULTS

Sample site selection. The location of the three sites chosen as permanent monitoring stations in Totten Inlet and Holmes Harbor are provided in Figures 2 and 3.

Comment [RE7]: Location of sampling sites should be placed in the Methods section

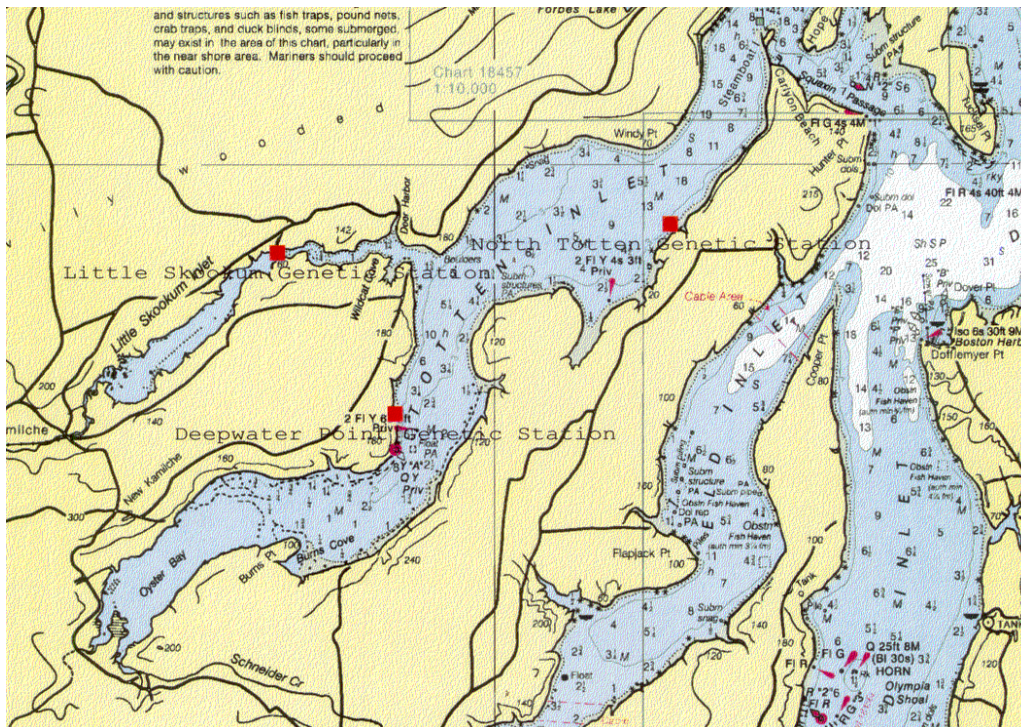


Figure 2. Mussel sample site locations in Totten Inlet examined during a 2002 survey.

Comment [RE8]: Not real easy to read the sample site legends on the chart, so short of redoing the chart, you could reiterate the sample site names in the figure legend.



Figure 3. Mussel sample site locations in Holmes Harbor examined during 1996 and 1997.

Totten Inlet. The Deepwater Point site is adjacent to an existing mussel farms where >450 metric tonnes of *M. galloprovincialis* are harvested each year from raft cultures. The beach was searched for ca. 300 m on either side of the farm for intertidal mussels. However, too few mussels were found to allow for a random survey. Mussels resembling *M. galloprovincialis* were collected from a fallen log and the random samples were collected from a shellfish predator net installed to protect oysters from starfish and crabs (Figure 4a).

Comment [RE9]: Again, the location descriptions, and description of how and what samples were taken, should be placed in the Methods section

Mussels were not found in sufficient quantities for a random survey in intertidal areas at the North Totten Inlet site adjacent to a proposed mussel culture site, which is north of the Gallagher Cove mussel farm. However, mussels dominated the fouling community located on piling adjacent to the beach (Figure 4b). The non random sample was collected from one piling and the random sample from a second piling.

A control station was established in Little Skookum Inlet. Large numbers of mussels apparently set on gravel bars in this inlet on a sporadic basis. However, all of these populations

appear to die off during their second summer of life and no intertidal populations were located in 2002. Random and non-random samples were collected from a concrete oyster dike at the location described in Figure 3c.



a) Deepwater Point predator fence b) North Totten piling c) Little Skookum concrete dike

Figure 3. Permanent sample sites located in Totten Inlet. a) Predator net adjacent to the Deepwater Point mussel farm; b) Piling adjacent to the proposed North Totten mussel farm and ca. 2.0 nm north of the Gallagher Cove farm; and c) the concrete oyster dike used as a reference location in Little Skookum Inlet.

Holmes Harbor. June Beach is a residential site with a personal use pier. It was located across the bay from an existing mussel farm. The creosote treated pilings host an abundant fouling community including a large population of mussels. A dense colony of intertidal mussels, covering approximately 1200 ft², surrounded these pilings. A 3 m square quadrat was laid out over the intertidal population for random sampling. Non random samples were collected from the piling. A second permanent sampling station was established in a scattered population of intertidal mussels found in a boulder field on Honeymoon Beach immediately adjacent to the mussel farm. Numerous randomly selected Cartesian coordinates were required in each year to collect the required 50 mussels for electrophoresis. Taylor’s Pier was selected as the third sample site because mussel seed from the hatchery is transshipped from trucks to boats for delivery to the farm at this location and there is a potential for the loss of seed. Sediments, in which large numbers of *Callianassa californiensis* were resident were too soft to support *Mytilus* sp. A 3 m square vertical sampling grid was established on a concrete bulkhead that was covered with mussels at this location.

Comment [RE10]: This paragraph describes methods used in a prior study, which is used lay a basis for discussion of the current study - I do not see the reasoning for including this in the results section of this paper.

Genetic analysis.

1996 - 1997 random sampling in Holmes Harbor. In 1996, a total of 53 mussels appeared to be *M. galloprovincialis* in the non-random samples. Of these, 26 were *M. galloprovincialis*, 26 were determined to be hybrids and one was *M. trossulus*. Fewer living *M. galloprovincialis* like mussels were observed in 1997 following a rainy winter in which surface salinity in Saratoga Passage and Holmes Harbor was significantly reduced. Numerous empty valves resembling *M. galloprovincialis* were observed. A search of several thousand feet of beach revealed only 12 mussels having *M. galloprovincialis* morphology in 1997. Two of these were hybrids and the remainder revealed only *M. galloprovincialis* alleles at the PGM-2 locus. The results of random sampling by year and sample location are provided in Table 1. In 1996,

Comment [RE11]: By visual observation of shell conformation, presumably, so please state explicitly, if that is the case.

2.8% of the randomly sampled mussels contained *M. galloprovincialis* alleles. In 1997, the proportion decreased to 1.4%.

Table 1. Results of random sampling of feral mussel populations at three locations in Holmes Harbor, Washington during 1996 and 1997. The number of each genotype is provided with the frequency of occurrence in parentheses

1996				
Site	Number	<i>M. trossulus</i>	<i>M. galloprovincialis</i>	Hybrids
Honeymoon Beach	48	47 (0.98)	1 (0.02)	0 (0.00)
Taylor Pier	48	47 (0.98)	1 (0.02)	0 (0.00)
June Beach	48	46 (0.96)	0 (0.00)	2 (0.04)

1997				
Site	Number	<i>M. trossulus</i>	<i>M. galloprovincialis</i>	Hybrids
Honeymoon Beach	50	50 (1.00)	0 (0.00)	0 (0.00)
Taylor Pier	49	48 (0.98)	0 (0.00)	1 (0.02)
June Beach	50	50 (1.00)	0 (0.00)	1 (0.00)

2002 random sampling in Totten Inlet. Results of the DNA analysis were reported by Gaffney (2003). A total of 184 mussels were typed for three nuclear markers. For 175 individuals, all markers were consistent, identifying mussels as *M. trossulus*, *M. galloprovincialis*, or hybrids. Nine individuals had hybrid genotypes at one or two loci, but single-species genotypes at the remaining locus or loci. These nine genotypes were confirmed by repeating the analysis from PCR to restriction enzyme digestion and evaluating the restriction digests on high-resolution precise polyacrylamide gels (Bio-Rad Criterion, 5% TBE). All genotypes were confirmed, indicating that these individuals were likely backcross hybrids, which were less common than putative F₁ hybrids (individuals with hybrid genotype at each locus; N = 42). Summary statistics describing these populations are provided in Table 2.

Comment [RE12]: Unclear what is meant by "all markers were consistent"

Comment [RE13]: Methods section "confirmation of genotypes"

Table 2. a) Genotypes observed in non-random samples of *Mytilus* collected in Totten Inlet. b) Summary results describing the genotypes of randomly collected mussels at two locations near raft cultures of *M. galloprovincialis* and at a control station in Totten Inlet, Washington. The frequency of *M. galloprovincialis* in the sampled population is provided in parentheses.

Comment [RE14]: It is not clear what the point of including the non-random, visual examination, results is in this section which is entitled "2002 random sampling in Totten Inlet" Please state why these are included and what conclusion or point they support.

a. Non-random samples with *M. galloprovincialis* shell morphology

Location	Number	<i>M. trossulus</i>	<i>M. galloprovincialis</i>	Hybrid	Apparent Back-cross hybrid
Deepwater Point	30	12 (0.40)	2 (0.07)	15 (0.50)	1 (0.03)
North Totten	30	16 (0.53)	9 (0.30)	1 (0.03)	4 (0.13)
Little Skookum Inlet	30	2 (0.07)	5 (0.17)	20 (0.67)	3 (0.10)

b. Random samples of *Mytilus* sp.

Location	Number	<i>M. trossulus</i>	<i>M. galloprovincialis</i>	Hybrid	Apparent Back-cross hybrid
Deepwater Point	34	32 (0.94)	1 (0.03)	1 (0.03)	0 (0.00)
North Totten	30	28 (0.93)	0 (0.00)	1 (0.03)	1 (0.03)
Little Skookum Inlet	30	27 (0.90)	0 (0.00)	3 (0.10)	0 (0.00)

DISCUSSION

Environmental niches associated with M. trossulus and M. galloprovincialis.

In Washington State, intertidal populations of mussels are rarely found outside areas of significantly reduced salinity associated with streams and rivers, which inhibit many stenohaline predators. *Mytilus galloprovincialis* does not tolerate reduced salinities of 15 to 20 ppt for extended periods of time and therefore does not share these sanctuaries with the low salinity adapted *M. trossulus*. Mussels find additional refuges from predators at high intertidal elevations on rocks, fallen logs, tree branches and near mean high water on piling, rocks, and manmade structures. Mussels also find refuge from predators on floating docks and buoys which is where many of Puget Sound collections have been made. Mussel veliger larvae remain in the water column for two to four weeks prior to metamorphosing to the pediveliger stage and settlement (Bayne, 1965). In many cases, currents disperse the larvae over large areas. However, there are areas like Penn Cove on Whidbey Island, where larvae are retained locally or are aggregated by currents. Anderson *et al.* (2002) concluded that the circulation patterns in Totten Inlet retain larvae within the inlet. Consistent with this observation, Brooks (2005b) found large numbers of 0.5 to 1.5 cm valve length Olympia oysters set on cultured mussels at the head of Totten Inlet. These larvae were likely spawned locally as Totten Inlet has historically supported a large population of this species.

Taxonomic status of M. galloprovincialis and its distribution in the Puget Sound.

Taxonomic status in Puget Sound. A careful reading of the literature suggests that the taxonomic status and origin of *M. galloprovincialis* in the Northeast Pacific is uncertain. Other than the observation of similar morphologies and allele frequencies at 8 loci, McDonald and Koehn (1988) did not present evidence supporting their assertion that *M. galloprovincialis* was an “invading” mussel introduced from Europe. The repeated reference to this assertion as a finding of fact is an example of how hypotheses become accepted knowledge based on the number of times the hypothesis is repeated. In contrast, the findings of Kenchington *et al.* (1995) suggest that *M. galloprovincialis* mussels in the Atlantic and Pacific Oceans diverged millions of years before the present. Their work does not contradict the potential for additional recent introductions of Mediterranean *M. galloprovincialis* in ballast water or as fouling organisms on the hulls of ships. However, it does indicate that the sample of Washington State *M. galloprovincialis* collected from a population of cultured mussels was not genetically similar to the Mediterranean sibling carrying the same name. Kenchington *et al.* (1995) found that cultured mussels resembling *M. galloprovincialis* in Washington State were genetically more distant from Mediterranean *M. galloprovincialis* than was *M. trossulus*. The fact is that at the present time we do not know the origin of *M. galloprovincialis* like mussels found in California and cultured in Washington State. It appears as likely that Pacific and Atlantic populations of blue mussels originated in the Pacific and migrated into the Atlantic millions of years ago. Alternatively, the small genetic and morphological differences seen in this sibling complex today could well be the result of environmental selection as suggested by Theisen (1978), Gartner-Kepkay *et al.* (1982), Tedengren *et al.* (1990), Johannesson *et al.* (1990) or Kautsky *et al.* (1990). What is known in Washington State is that when we developed the ability to genetically distinguish these siblings and looked in Puget Sound, Brooks (1991) reported *M. galloprovincialis* during a 1988 survey of raft cultures that were initiated in 1985 and in a feral

population containing up to six year old *M. galloprovincialis* from Dyes Inlet, Washington. Additional specimens were found in Sequim Bay on the Straits of Juan de Fuca.

Comment [RE15]: Author, please clarify if your point is that the described mussels from this collection predated mussel culture or was spatially removed from mussel culture at that time, or other point intended by this statement.

Distribution in Puget Sound. Wonham's (2004) review described *M. galloprovincialis* alleles in mussels collected at 23 of 68 sites previously reported in the literature with *M. galloprovincialis* frequencies as high as 0.35 at Silverdale and hybrid frequencies as high as 0.67 at the same location. However, it must be emphasized that excepting Brooks (1996, 1997), the cited surveys were not designed to randomly sample mussel populations. The random sampling designs of Brooks (1996, 1997) suggest that feral mussel populations in Holmes Harbor contained zero to 2.0% *M. galloprovincialis* and up to 4% *M. galloprovincialis* x *M. trossulus* hybrids. In 2002, feral mussel populations in Totten Inlet contained between zero and 3% *M. galloprovincialis* and zero to 10% hybrids. Thus it appears that in Totten Inlet, where intensive *M. galloprovincialis* culture has been practiced for 20 years that between 3 and 10 percent of the feral mussel population may contain *M. galloprovincialis* genes.

Potential for M. galloprovincialis to displace or genetically pollute M. trossulus stocks in Puget Sound. Based on the report of McDonald and Koehn (1988), several authors have asserted that *M. galloprovincialis* has invaded and displaced local populations of *M. trossulus*. However, the often quoted report of McDonald and Koehn (1988) was simply a poorly substantiated hypothesis. The more substantive report of Kenchington *et al.* (1995) indicates a greater genetic divergence between Mediterranean *M. galloprovincialis* and Washington State cultured *M. galloprovincialis* that exists between *M. trossulus* and the Mediterranean mussel. From a physiological point of view, the work of Brooks (1991) suggests that populations of *M. trossulus* suffers nearly 100% mortality at temperatures above 20 °C and that sustained populations would be unlikely in the warm waters of Southern California where the warm water and constantly high salinity adapted *M. galloprovincialis* currently thrives. In contrast the relatively cold waters of Puget Sound experience large reductions in salinity – particularly during the winter peak spawning period of *M. galloprovincialis* which would likely inhibit, but not extinguish, successful recruitment.

Conclusions

It is likely that boat traffic and aquaculture will continue as potential sources of blue mussel sibling species along the Pacific Coast as described by Wonham (2004). However, the environmental niches preferred by these two siblings are quite different and it appears reasonable to assume that *M. galloprovincialis* will continue to dominate mussel populations in more southerly latitudes and that *M. trossulus* will dominate in more northerly latitudes. This hypothesis is supported by the existing population structures of these mussels along the Pacific Coast including the existing zone of sympatry and hybridization found between San Francisco and Humboldt Bay in California. The non-random sampling reported herein indicates that mussel siblings can be distinguished in mixed populations with reasonable reliability, making non-random or haphazard sampling appropriate for determining the presence – absence of *M. galloprovincialis*. However, these results also imply that random sampling designs must be used to estimate population parameters. It should be emphasized that the *M. galloprovincialis* allele frequencies reported herein were determined in two Puget Sound areas where intensive *M. galloprovincialis* culture is conducted and it would be inappropriate to apply these results to mussel populations located outside these areas.

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